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pH Dependence of the Equilibrium Parameters of the Reaction of 5,5′-dithiobis(2-nitrobenzoate) with Dog Carbonmonoxyhaemoglobin at 450 nm

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Abstract:
This study was undertaken to determine the dependence of equilibrium constants of the reactions of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the sulphydryl groups of dog carbonmonoxyhaemoglobin on pH, and the effect of organic phosphates on these equilibrium constants at 450 nm. The equilibrium constants showed strong pH dependence varying about three orders of magnitude for stripped dog carbonmonoxyhaemoglobin and about four orders of magnitude for haemoglobin in the presence of inositol hexakisphosphate (inositol-P_6, an organic phosphate) between pH 5.6 and 9.0. The values of K_{rt}, the equilibrium constant for the r t tertiary structure transition, are 0.33 and 2.28 for stripped haemoglobin and haemoglobin in the presence of inositol-P_6, respectively. The corresponding ***t****-isomer population are 24.76% and 73.82%. The comparability study showed that inositol-P_6 had no effect on the affinity of dog carbonmonoxyhaemoglobin for DTNB at all experimental pHs.*

Keywords*: pH dependence, DTNB, Dog, Haemoglobin, Inositol-P_6*

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I. Introduction

Haemoglobin (Hb), a tetrameric protein found in vertebrate erythrocyte, is an iron containing protein that conveys oxygen in the blood. Oxygen is temporarily stored before use in the tissues by a related but less complex protein called myoglobin. Ninety nine percent of the cellular oxygen requirements are met by this molecule while diffusion accounts for only one percent. Both haemoglobin and myoglobin are conjugated proteins. They are found in the red blood cells of vertebrates such as mammals, reptiles, birds, amphibian and fish. Haemoglobin is also found in the plasma of some invertebrates such as annelid and worms. Mammalian haemoglobin is the most efficient [1]. Haemoglobin comprises of two parts: the haem portion ferroprotoporphyrin, which is the non-protein portion and the protein portion called the globin. The globins of various haemoglobins differ from one animal to another. They consist of four polypeptide chains; α and β identical pairs, stabilized by weak secondary forces such as hydrogen bonds, non-polar interactions and interpeptide salt linkages called salt bridges [2].

Sulphydryl groups are present as cysteine residues of proteins. They are known to catalyze or inhibit many reactions in living systems [3], when a sulphydryl group in an enzyme is charged. The high reactivity of the sulphydryl groups has placed them among the most important functional groups to be considered when investigating the reactivities, functions, mechanisms and conformations of macro-molecules. The CysF9[93]β sulphydryl group has been used to study the change in tertiary and quaternary structures haemoglobin [4-9]. Thiols play a principal role in maintaining oxidation–reduction states of proteins, cells and organisms. However, the susceptibility of thiols to oxidation can lead to the formation of disulphides and higher oxidation products often with the loss of biological activities [10].

Haemoglobin sulphydryl groups are some of the most studied amino acid residues [11]. Different haemoglobins have different numbers of sulphydryl groups. The number of sulphydryl groups in haemoglobins, determined by spectrophotometric titrations with sulphydryl sensitive reagents have been found to be generally less than the total number of cysteines in the molecule, since some thiols are masked at the sub-unit interfaces or hidden in hydrophobic regions and are not accessible to sulphydryl reagents. The number of titratable thiol groups in haemoglobin depends on the thiol reagent used [11]. Mercurial reagents react with all free thiol groups in haemoglobin while non-mercurial reagents titrate only those thiols which can form the thiolate anion [12,13].

Reagents that react with sulphydryl groups are called sulphydryl reagents. Examples of sulphydryl reagents are iodoacetamide, iodoacetate, maleimide, p-chloromercuri(II) benzoate (p-CMB), DTNB, bis (p-

nitrophenyl) disulphide, 2,2′-dithiobis pyridine and 4,4′-dithiobis pyridine. Most sulphydryl reagents are not stable at room temperature and are to be stored at low temperature. The sensitivity of DTNB (Fig. 1) to its environment enhances its use as sulphydryl reagent. It is also commercially available and reasonably soluble in water as the salt, it is also stable in neutral solution if protected from light.

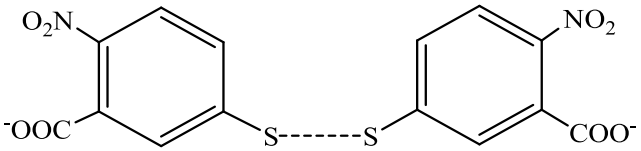

Figure 1: *Structure of 5,5′-dithiobis(2-nitrobenzoate acid) (DTNB)*

The sulphydryl group reactivity is dependent on the neighbouring amino acid residue. The ionization of the charged amino acids in haemoglobin depends on pH. The sulphydryl group must be thiolated to react with non-mercurial sulphydryl reagents. Sulphydryl group reactivity increases with positive charge residues and decreases with negative charge residues therefore increasing the value of the *pQ* (ionization constant) [11]. The pH dependence studies of the reactivities of these sulphydryl groups are useful in the determination of the number and the nature of the amino acid residues influencing the reactivity of such sulphydryl groups. The pH dependence profile nature is determined by the ionization constants of the charged amino acid residue, the sulphydryl reagents used and the distance of the charged residues from the reacting sulphydryl group. The values of the *pQ* are dependent on the ionic strength, group and the temperature. Studies of the reactivity of sulphydryl group with respect to pH have shown that there are three types of pH dependence profile.

The titration of dog (*Canis familiaris*) haemoglobin with DTNB shows that two sulphydryl groups per molecules are accessible towards DTNB. These are located at the F9[93]β and G14[118]β positions. The aims of this research are to determine the equilibrium constant, K_{eq} of the reaction of DTNB with dog carbonmonoxyhaemoglobin at 450 nm, study the profile of K_{eq} as a function of pH for the reaction and to determine the effect of organic phosphates on the reaction of DTNB with dog carbonmonoxyhaemoglobin at the pH range (5.6 – 9.0).

II. Experimental

All chemicals used were of analytical grade. All samples and solutions used, which include preparation of dog haemoglobin, carbonmonoxy derivative of the dog haemoglobin, buffer solutions, isotonic saline and dialyzing solutions, DTNB and inositol-P_6 solutions, and the resins for the Dintzis column were prepared using the Beetlestone's haemoglobin laboratory procedures [14].

Determination of Carbonmonoxyhaemoglobin Concentration

A 0.1 ml of the carbonmonoxyhaemoglobin was mixed with 10 ml of distilled water. The absorbance of the diluted carbonmonoxyhaemoglobin was taken at 537.5 nm with the filter set at zero using distilled water as reference. The concentration was determined with the aid of Eq. (1).

$$C = \frac{A_{537.5}}{\varepsilon l} . \frac{V + v}{v} \qquad (1)$$

In Eq. (1), C is the concentration of the dog carbonmonoxyhaemoglobin in μmol (haem) dm^{-3}; $A_{537.5}$ is the absorbance at 537.5 nm; ε represents molar extinction coefficient (14,000 cm^{-1} mol^{-1} dm^3); l is the path length of cuvette used; V is the volume of the distilled water; and v is the volume of dog carbonmonoxyhaemoglobin

Equilibrium parameters determination for the reaction of DTNB with sulphydryl groups

A 50 μmol (haem) dm^{-3} of the dog carbonmonoxyhaemoglobin solution was prepared in buffer of specific pH with ionic strength of 50 mmol dm^{-3}. A 3 ml each of this solution was measured accurately using a micropipette into eleven test-tubes, increasing volumes (10 – 100 μl) of stock 29.07 mmol dm^{-3} DTNB were added to the tubes.. These mixtures were left to equilibrate for 6 h at 25°C in a thermostated water bath. DTNB was not added to reference sample.

The absorbance of each solution was taken at 450 nm on a JENWAY 754 UV – VIS spectrophotometer. The concentration of the 5-thio-2-nitrobenzoate (TNB^-) produced from the reaction of DTNB was calculated from the change in absorbance, assuming a molar extinction coefficient of 14,000 cm^{-1} mol^{-1} dm^3 for TNB using a micro-math software. The equilibrium constant (K_{eq}) was also calculated for each test-tube using the micro-math scientist software. The average value of K_{eq} was calculated with a standard error < 20%. pH of the resulting mixture was measured at the end of the incubation period using a pH 5.3C precise pH meter. This procedure was repeated for phosphate buffers pH 5.6 – 8.0 and borate buffers pH 8.0 – 9.0. The

negative logarithm to base ten of the equilibrium constants were calculated and then plotted against the corresponding pH.

For the experiment in the presence of inositol-P_6, a 50 µmol dm^{-3} of inositol-P_6 was added before equilibration.

III. Results And Discussion

Stripped dog carbonmonoxyhaemoglobin

Equilibrium reaction of DTNB with the sulphydryl groups of dog carbonmonoxyhaemoglobin was investigated in absence of inositol-P_6. The data for the negative logarithm to base ten of the equilibrium constant ($-\log_{10}K_{eq}$) at the various pH for the reaction is shown in Fig. 2. From this plot, it is shown that the equilibrium constants show strong pH dependence varying over about three orders of magnitude between pH 5.6 and 9.0.

Equilibrium reaction of DTNB with dog carbonmonoxyhaemoglobin in the presence of inositol-P_6

Fig. 3 shows the profile of $-\log_{10}K_{eq}$ against various pH values for the reaction of DTNB with the two titratable sulphydryl groups of the carbonmonoxy derivative of dog haemoglobin in the presence of inositol-P_6. The equilibrium constants also show strong pH dependence varying over about four orders of magnitude between pH 5.6 and 9.0.

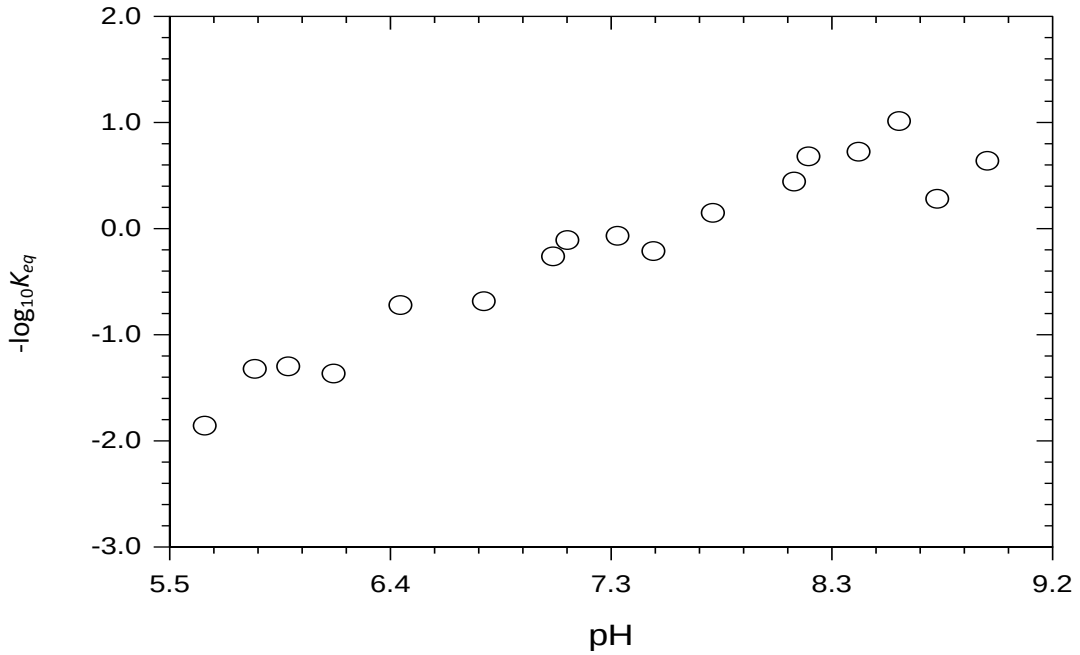

Figure 2: Dependence of the $-\log_{10}K_{eq}$ on pH of the reaction of stripped carbonmonoxy derivative of dog haemoglobin with DTNB at 25ºC. Extinction coefficient of 1073 cm^{-1} M^{-1} was assumed for the TNB^- at the operating wavelength of 450 nm

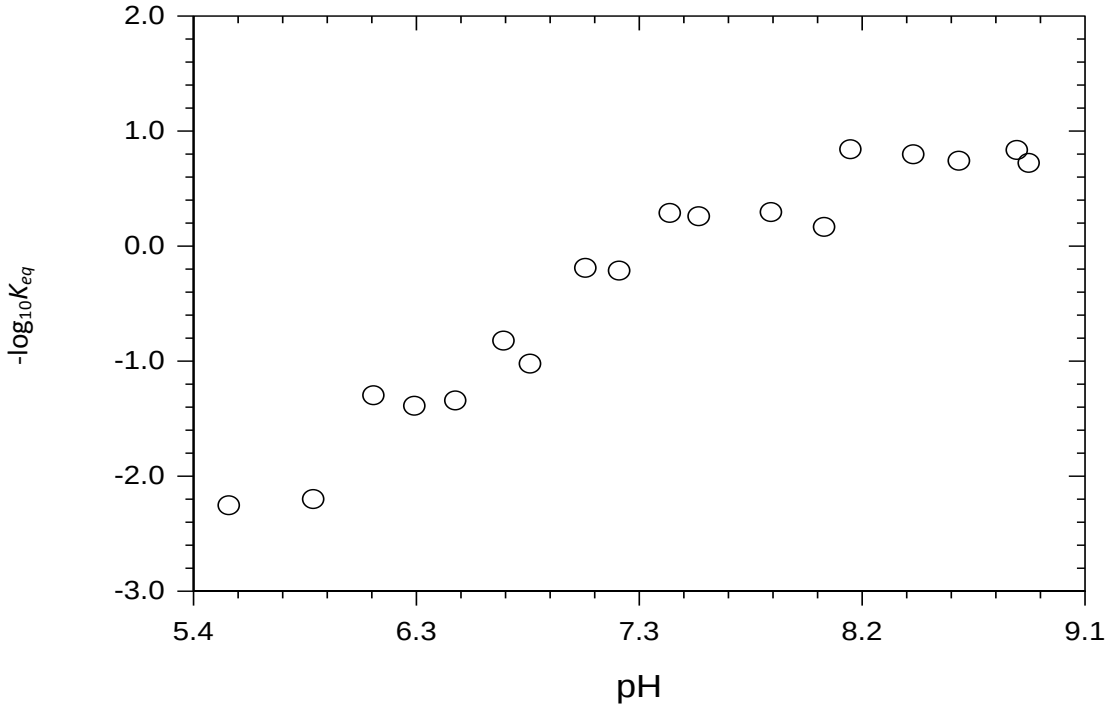

Figure 3: Dependence $-\log_{10}K_{eq}$ on pH of the reaction of dog carbonmonoxyhaemoglobin with DTNB in the presence of inositol-P_6 at 25ºC. Extinction coefficient of 1073 cm^{-1} M^{-1} was assumed for the TNB^- at the operating wavelength of 450 nm.

Comparison of the pH dependence of the reaction of DTNB with and without inositol-P_6

Fig. 4 shows the comparability plot of the stripped Hb and Hb in the presence of inositol-P_6. The empty circles represent data in the absence of inositol-P_6 while the filled circles show results in the presence of inositol-P_6.

Table 1: *Best-fit parameters employed to fit the equilibrium data reported in Figs 1 and 2*

	pQ_{1r}	pQ_{2r}	pQ_{1t}	pQ_{2t}	K_{E3}	K_{rt}
Stripped Hb	5.95	6.89	5.81	8.23	0.35	0.33
Hb in the presence of inositol-P_6	5.65	6.50	6.09	8.43	0.16	2.82

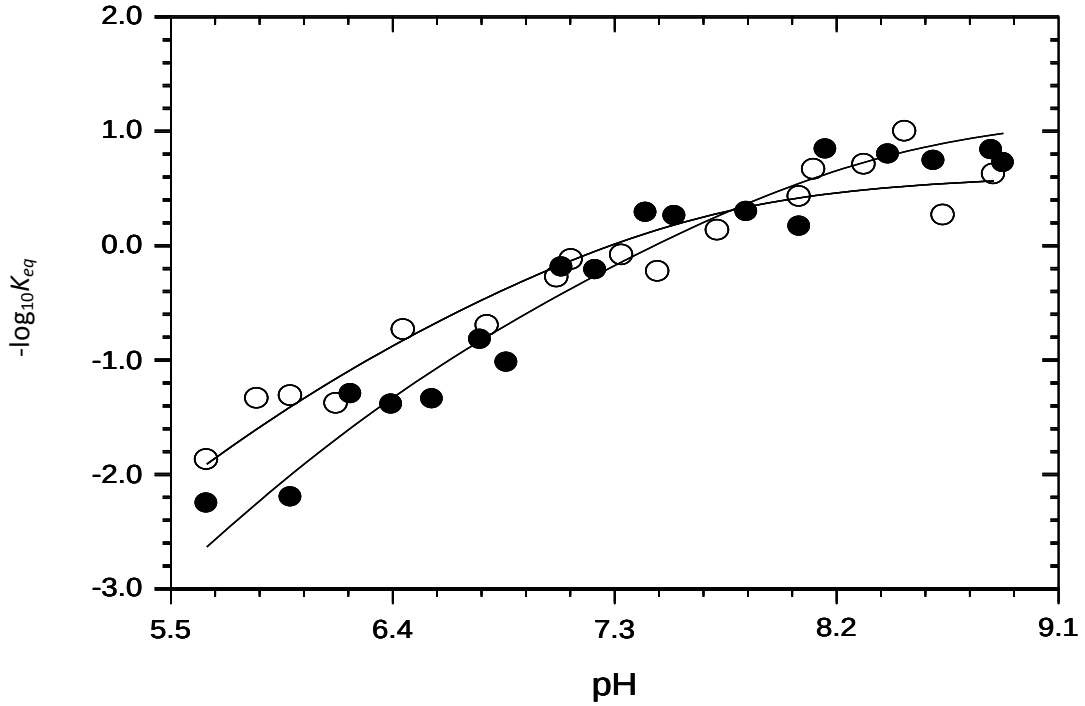

Figure 4*: Comparability of the individual plots, Stripped Hb and Hb with inositol-P_6.*

There are two ionizable groups in the *r* isomer (pQ_{1r} and pQ_{2r}) and two ionizable groups in the *t* isomer (pQ_{1t} and pQ_{2t}). The values of pQ_{1r}, pQ_{2r}, pQ_{1t}, pQ_{2t}, K_{rt} and K_{E3} are shown in Table 1. The value of K_{rt} of stripped carbonmonoxyhaemoglobin is 0.33; this gives a value of 24.8% for the relative population of the *t* isomer. The value of K_{rt} in the presence of inositol-P_6 is 2.82; this gives a value of 72.8% for the relative population of the *t* isomer. It is seen in Table 1 that inositol-P_6 increases K_{rt} value by a factor of 8.5 but has negligible effects on the *pQs* of coupled ionizable groups and K_{E3}, the equilibrium constant at the highest pH.

Theoretical aspect of the equilibrium study

It is known that DTNB reacts only with the thiolate anion form of a sulphydryl group. The reaction of DTNB with haemoglobin can be represented by Eq. (2) [11].

$$HbSH + DTNB \overset{K_{SH}}{\rightleftharpoons} H^+ + HbS^- + DTNB \overset{K_{eq}}{\rightleftharpoons} H^+ + HbS.TNB + TNB^- \overset{K_{TNB}}{\rightleftharpoons} HbS.TNB + TNBH \quad (2)$$

K_{SH} is the ionization constant of the sulphydryl group, K_{eq} is the equilibrium constant and K_{TNB} is the ionization constant of TNBH.

The ionization constant of the sulphydryl group is given by Eq. (3).

$$K_{SH} = \frac{[H^+][HbS^-]_f}{[HbSH]_f} \quad (3)$$

The ionization constant of TNBH is given by Eq. (4).

$$K_{TNB} = \frac{[H^+][TNB^-]}{[TNBH]} \quad (4)$$

The equilibrium constant is given by Eq. (5).

$$K_{eq} = \frac{[HbS.TNB][TNB^-]}{[HbS]_f[DTNB]_f} \quad (5)$$

The subscript *f* in the above equations represents 'unreacted species'.

K_{eq} is given by Eq. (6).

$$K_{eq} = \frac{[TNB^-]^2 \left\{1 + \frac{[H^+]}{K_{TNB}}\right\}\left\{1 + \frac{[H^+]}{K_{SH}}\right\}}{\left\{[HbS]_f - [TNB^-]\left\{1 + \frac{[H^+]}{K_{TNB}}\right\}\right\}\left\{\left([DTNB]_{total} - [TNB^-]\right)\left\{1 + \frac{[H^+]}{K_{TNB}}\right\}\right\}} \quad (6)$$

It is clear from Eq. (6) that if the total haemoglobin and DTNB concentrations are known, K_{eq} for the DTNB reaction with haemoglobin can be determined, provided the ionization of TNBH, K_{TNB} and that of the sulphydryl groups, K_{SH} are known. The pK_{SH} of the CysF9[93]β sulphydryl group has a value of ≈8.3 while that of pK_{TNB} is 5.27 [15].

Examination of Eq. (2) shows that the reaction of the haemoglobin with DTNB consists of the opposing second order processes: $HbS + DTNB \rightarrow HbS.TNB + TNB^-$ (forward reaction) and $TNB^- + HbS.TNB \rightarrow DTNB + HbS$ (reverse reaction). The forward reaction is easy to follow experimentally. However, the direct experimental measurement of the kinetic parameters of the reverse reaction is not feasible. This is generally true of most opposing second order reactions. Since K_{eq} and K_f (forward reaction rate constant) can be determined as a function of pH, it becomes possible to determine K_r (reverse reaction rate constant) as a function of pH.

It has been demonstrated in kinetics that the reaction of DTNB with the haemoglobins of the dog is reversible [16]. From the results obtained in this study, it is seen that the reaction of DTNB with the sulphydryl groups of the dog haemoglobin, both with and without inositol-P_6 is reversible and pH dependent. The determination of the equilibrium constants, K_{eq} of different pH for the reaction of DTNB with the sulphydryl groups of the dog haemoglobin is an important parameter because it enables the determination of the second order rate constant for the reverse of the DTNB reaction, K_r, which is otherwise not possible by direct experimental measurement.

Furthermore, the knowledge of the equilibrium constant, K_{eq}, has the great benefit because it enabled the determination of the apparent forward second order rate constant, K_f, without the necessity of doing a concentration dependence of K_{obs}, the pseudo-first order rate constant. From the comparability study, it is also shown that the affinity of dog carbonmonoxyhaemoglobin for DTNB is not affected by the presence of inositol-P_6.

IV. CONCLUSION

From the results obtained in this study, it is shown that the reactions of DTNB with the sulphydryl groups of the dog carbonmonoxyhaemoglobin, both with and without inositol-P_6 show strong pH dependence varying over about four orders of magnitude between pH 5.6 and 9.0. There is no appreciable difference in the variation in pH dependence of the equilibrium constants from the comparability plot of stripped Hb and Hb + inositol-P_6. This is an indication that there is no change in affinity of dog carbonmonoxyhaemoglobin for DTNB in the presence of the organic phosphate. It may be restated that oxygen affinity of stripped dog carbonmonoxyhaemoglobin will be the same as the one in the presence of inositol-P_6 if oxygen binding experiments are carried. Two groups were found ionizing in *r* and *t* isomers. Inositol-P_6 increased the value of K_{rt} by 8.5-fold.

Conflicts of Interest

The authors whose names appear below certify that they have NO affiliations with or involment in any organization or entity with any financial such as honoraria; educational grants or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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